PREFACE

The fields of assisted reproducton and nuclear transfer, while often controversial in their relatively brief lifetimes, have been and continue to be exceedingly dynamic. In *Assisted Fertilization and Nuclear Transfer in Mammals*, we have treated these subjects as a continuum since assisted sexual reproduction provides the technical groundwork for asexual reproduction by nuclear transfer and because both cytoplasmic and nuclear transfer protocols are in assisted reproductive technology (ART) clinical trial programs in this country. The basic reproductive physiology underlying these technological achievements holds the key to understanding the events comprising fertilization and early mammalian development, while providing treatment modalities for most cases of infertility. Our current efforts, detailed here, are predicated historically on the discovery in the late 1950s, that sperm must undergo a process called capacitation before fertilizing an oocyte and thereafter, on the development of strategies to recover and fertilize viable oocytes. Application in humans, pioneered by Drs. Patrick Steptoe and Robert Edwards in Cambridge, England culminated in 1978 with the birth of Louise Brown, which was followed by explosive growth in the clinical use of this technology that was encouraged by the high interest level of the infertility community. At present, the clinical ARTs are available worldwide with over 300 programs in the US alone; according to the American Society of Reproductive Medicine in 1995, 11, 315 women gave birth to children conceived by some form of ART. At the clinical level, we might trace this technological revolution from sperm isolation, cryopreservation, and capacitation, to in vitro fertilization, embryo cryopreservation, intracytoplasmic sperm injection, and extended embryo culture to the latest hot topics of in vitro oocyte maturation, oocyte cryopreservation, and cytoplasmic and nuclear transfer.

With regard to nuclear transfer in mammals, relatively undifferentiated embryonic cells have been used successfully as a source of the donor nucleus in a number of species beginning in the early 1980s and leading to application in the rhesus monkey in 1997. The revolutionary announcement in 1997 of somatic cell cloning by Ian Wilmut, Keith Campbell, and colleagues at the Roslyn Institute in Scotland opened the possibility that existing individuals could be reproduced asexually, and indeed that groups of genetically similar animals could be produced this way. In response to this discovery, the past two years has seen intense interest in the field, with confirmation of somatic cell cloning in other species and an ongoing debate concerning potential application in humans.

This volume, *Assisted Fertilization and Nuclear Transfer in Mammals*, is by design unique, for instead of writing principally for the ARTs practitioner, we have written for a greater audience including students, practitioners of the clinical ARTs, our colleagues responsible for animal care, and research scientists. Our objectives include: The provision of a historical perspective on the development and application of these technologies in animals that in many, but not all, instances preceded clinical application; the treatment of subjects from both a basic scientist's and a practicing clinician's perspective in an effort to encourage communication between these sometimes diverse groups; and the inclusion of updates on several of the more dynamic clinical areas, such as gamete and embyro cryopreservation, intracytoplasmic sperm injection, and oocyte in vitro maturation. In all cases, detailed bibliographies have been encouraged in an effort to provide

historical continuity for the student or for those desirous of additional insights and reading.

We would like to express appreciation to the distinguished authors who accepted our invitation to participate in this project, to Humana Press, and to Dr. P. Michael Conn, the series editor, for their confidence in our ability to organize and complete this project. We also thank Julianne White for her excellent editorial assistance.

Don P. Wolf
Mary Zelinski-Wooten

2 Advances in Animal In Vitro Fertilization

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CONTENTS

INTRODUCTION

Much progress of relevance to the union of mammalian gametes in laboratory conditions has been reported during the last half century. Early in this interval, it was realized that spermatozoa must undergo the process of capacitation and that conditions for gamete union must, at least crudely, resemble those found at the normal site of fertilization, i.e., within the oviduct. Today it is possible to initiate early embryonic development of just about any mammalian species by co-incubating homologous ova with treated spermatozoa. This review will trace the development of in vitro fertilization (IVF) technology through experimentation with several laboratory and domestic animals. Information regarding nonhuman primates appears in Chapter 16. A few of hundreds of possible examples from available literature reports will be mentioned to illustrate advances in this important area of biological science. Utilization of IVF for enhancing reproductive efficiency, especially for cattle, and in preservation of genetically valuable and endangered animal species promises to impact significantly on animal breeding strategies.

EARLY IVF IN LABORATORY ANIMALS

Rabbit

Independent reports in 1951 by Austin involving rats and rabbits *(1)* and by Chang in rabbits *(2)* described a need for sperm cells to undergo a physiological change—termed sperm capacitation *(3)*—in the female reproductive tract prior to penetrating an ovum.

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Three years later, Thibault et al. *(4)* reported that sperm capacitation was an essential prerequisite to fertilization of rabbit ova in vitro. Whether sperm cells were inadvertently capacitated to achieve authentic fertilization in vitro as suggested by a dozen or so earlier descriptive accounts involving rabbit, guinea pig, mouse, and rat gametes seems doubtful (for review, *see* ref. *5*), with the possible exception of reports of Moricard and Bossu *(6)* and Smith *(7)*. Independently, these authors found pieces of oviduct to be beneficial to sperm penetration through the rabbit zona pellucida in vitro.

Indisputable criteria for achievement of rabbit IVF including birth of live young were reported in 1959 by M.C. Chang *(8)*. This work, a first in mammals, involved exposure of recently ovulated ova to sperm taken from uteri of mated does, and later, transfer of cleaved ova to oviducts of hormonally synchronous recipient does that delivered live offspring. Similarly, birth of live offspring was achieved following transfer of rabbit embryos derived from IVF in several additional reports in the ensuing decade *(9–11)*, and by 1970, IVF was documented by microcinematography *(12)*. In addition to tubal ova, soon it was possible to fertilize ova recovered from the ovarian surface just after ovulation *(13)* and taken from ovarian follicles just prior to ovulation *(14,15)*.

It was recognized early in serious efforts to achieve IVF that best results followed rapid handling of gametes at near body temperature prior to insemination. Paraffin or silicone oil covering of media and high relative humidity assisted in maintaining constant temperature by preventing evaporation. An oxygen tension of 8% to resemble that measured within the oviduct *(16,17)* and 5% CO_2 to maintain proper pH of a bicarbonate-containing medium were found compatible with IVF *(18)*. Thus, a simple defined medium consisting of a salt solution with crystalline bovine albumin, glucose, and bicarbonate to maintain a pH of 7.8 to resemble estrous oviductal fluid *(19)* at 38ºC (rabbit body temperature) was found to consistently support sperm penetration of rabbit ova *(18)*. This, in turn, allowed conduct of controlled experiments to test various influences on the mammalian fertilization process *(5)*. Early work in the rabbit provided a background for facilitating extension of IVF technology to other mammalian species (e.g., cat, cow, goat, hamster, human, mouse, and so on). This work involved assessment of factors for their influence on proportions of ova that could be fertilized and other efforts to capacitate rabbit spermatozoa in vitro. Cleavage of resulting zygotes was consistently obtained following transfer into a serum-containing medium with a more neutral pH. From embryo culture experiments involving in vivo-derived zygotes and 2-cell embryos, it soon became clear that pyruvate was an important substrate for support of initial cleavage development *(20–22)*. The need for transfer of inseminated ova into another medium could be eliminated by addition of 10^{-5} M pyruvate to the defined medium, which already contained adequate concentrations of glucose and albumin to support later cleavage stages of preimplantation rabbit embryos *(23,24)*. This medium developed for rabbit IVF has been variously referred to as Defined Medium (DM) *(24)*, Brackett's medium *(25)*, and Brackett-Olphant (BO) medium *(26)*.

IVF provided an improvement over in vivo testing for sperm fertilizing ability. A clearer understanding of in vivo and in vitro influences on sperm could now be determined. In rabbits with surgically removed oviducts, spermatozoa were capacitated in uteri as demonstrated by fertilization of approx 90% of recently ovulated ova recovered from ovarian surfaces *(27)*. Optimal IVF results (90.6% fertilization) could also be duplicated after sperm capacitation for 17 h in uteri of hormonally treated does that had been ovariectomized and salpingectomized a month before. Hormonal treatments con-

sisted of intramuscular injections of an estradiol preparation for 6 d with a single progestin treatment at the time of mating *(28)*. Larger doses of progestin (than used in these experiments) inhibit capacitation of sperm in the uterus *(29)*. That uterine fluids produced under appropriate hormonal influences could effect capacitation was further demonstrated by IVF after sperm treatments with uterine fluid recovered from intact gonadotropin-treated does near the time of ovulation *(30)*. Rabbit epididymal sperm are more easily capacitated than are ejaculated sperm cells that have been exposed to additional male accessory gland secretions *(31,32)*. An important component of the capacitation of ejaculated sperm was shown by an immunological approach to involve removal or alteration of sperm surface proteins concurrent with increasing intervals of residence in the female reproductive tract *(33)*. It was further demonstrated that a seminal plasma glycoprotein with a molecular weight of approx 115,000 could be used to reversibly decapacitate uterine capacitated sperm *(34)*.

In other experiments, IVF was employed to assess acquisition of sperm fertilizing ability after membrane-altering sperm treatments, e.g., with lysolecithin *(30)*, or Sendai virus *(35)*. Following earlier leads, efforts to remove or alter sperm surface antigens (e.g., removal of decapacitation factor) involved incubation in DM prepared with additional NaCl, i.e., high ionic strength (HIS) medium (380 mOsm/kg). This led, by 1975, to achievement of in vitro capacitation combined with IVF and embryo transfer to obtain offspring, a mammalian first for ejaculated spermatozoa *(24)*. Variability in performance of spermatozoa from different bucks was recognized along with retardation in development of fertilization and early cleavage stages. The latter suggested delayed sperm penetration of ova. Various facets of this work were soon repeated in other laboratories *(36–38)*. Improvement in proportions of ova fertilized and in more normal temporal development followed application of this approach for IVF with cauda epididymal spermatozoa *(32)*. With additional incubation of HIS- or DM-treated epididymal spermatozoa prior to in vitro insemination, sperm penetration was almost complete by 3.25 h after insemination *(38)*, temporally comparable to in vitro penetration by spermatozoa capacitated in vivo *(12)*. Contemporaneous reports from several laboratories pointed out that ejaculated rabbit spermatozoa capacitated in vitro were not entirely equivalent to in vivo capacitated spermatozoa in fertilizing ability *(39–41)*. However, with 12–22 h of preincubation after HIS treatment, in vitro capacitated (ejaculated) spermatozoa rapidly penetrated ova and initiated development that was temporally comparable to that following in vitro insemination with in vivo capacitated uterine spermatozoa *(42)*. The desirabililty of removing inseminated ova from spermatozoa as quickly as possible *(27)* was confirmed. By transferring oocytes into fresh DM 6 h after insemination, fertilization results improved from 44% (17 of 39 oocytes) to 85% (40 of 47 oocytes) and development to the 4-cell stage improved from 35% (6 of 17) to 78% (31 of 40). In these experiments averages of 57% total and 29% progressive motility scores were noted for spermatozoa at insemination. Evidence from IVF and in vivo fertilization in test does documented an approximation of in vitro capacitation and IVF with the normal in vivo processes when the ejaculated spermatozoa were incubated for additional intervals after HIS treatment *(42)*. In this work, 43 of 52 embryos (83%) resulting from IVF of follicular, surface, and tubal oocytes, collectively, reached morula or blastocyst stages *(42)* following culture for 78–142 h post-insemination in serum-supplemented Ham's F-10 *(43)*. The proportion of 4-cell stage embryos (resulting from fertilization with in vitro capacitated spermatozoa) that developed into normal offspring was comparable to embryo transfer results after IVF

with in vivo capacitated spermatozoa in previous work *(15)*. Despite functional results, there is no assurance that mechanisms of such in vitro treatments duplicate those that normally occur in vivo.

Hamster

In 1963 and 1964, Yanagimachi and Chang *(44,45)* reported initial success in fertilizing rodent ova in vitro along with in vitro capacitation of hamster epididymal sperm. Hamster sperm heads and male pronuclei with tails of fertilizing spermatozoa were found within ooplasm after incubation of ova with epididymal sperm in Tyrode's solution under paraffin oil. Spermatozoa recovered from the uterus of females mated 4–5 h before fertilized 134 (64.7%) of 207 ova, uterine sperm recovered 0.5 h after mating fertilized 52 of 100 ova, and epididymal sperm fertilized 80 (44.4%) of 180 ova. Although more rapid penetration of ova correlated with uterine exposure of sperm recovered after mating their experiments with epididymal sperm marked initial success with in vitro capacitation and IVF in a single system. Yanagimachi *(46)* described microscopic observation of sperm penetration to include absence of the outer acrosome membrane before the spermatozoon began penetrating the zona pellucida. Penetration was at an angle to the zona surface and required 3–4 min. Then, in less than 2 s, the sperm head lay flat on the oolemma and, without further motility, the spermatozoon sank into the vitellus.

Barros and Austin *(47,48)* found a higher incidence of polyspermy after insemination (with epididymal spermatozoa) of follicular oocytes recovered just before ovulation than for tubal ova. Both follicular fluid and tubal fluid supported the hamster sperm acrosome reaction in vitro *(47)*. Follicular contents recovered 9–10 h after human chorionic gonadotropin (hCG) injection induced the acrosome reaction in 97% of epididymal spermatozoa, whereas follicular contents recovered 4 h after hCG induced the acrosome reaction in only 43% of epididymal spermatozoa, suggesting accumulation of an active agent in the follicle in the preovulatory phase. After epididymal sperm incubation for 3 h with Tyrode's supplemented with follicular fluid sperm penetration of ova usually occurred 30–50 min after insemination *(49)*. Capacitation took place most efficiently with hamster follicular fluid present, but mouse follicular fluid was found to be better in this regard than rat follicular fluid, whereas rabbit follicular fluid was totally ineffective *(49)*. Bovine follicular fluids also induced the hamster acrosome reaction *(50)*. Two fractions of this fluid were implicated in capacitation *(50)*, a dialyzable and heat stable (at 90°C) fraction with sperm-activating properties, and a nondialyzable and heat labile (at 90°C) fraction responsible for induction of the acrosome reaction. Similar activities were found in bovine serum. The large molecular weight fraction was identified as albumin *(51)*. Data on electrophoretic components of hamster uterine fluid indicated an increase in albumin at the appropriate time for a physiological role in preparing spermatozoa for fertilization *(52)*. Meizel et al. *(53)* recognized important influences of taurine and hypotaurine found in sperm and reproductive tract fluids on hamster sperm motility, capacitation, and the acrosome reaction in vitro.

Although 75% or more of inseminated ova could be fertilized routinely development beyond the 2-cell stage was not possible *(54)*. Development of "hamster embryo culture medium" (HECM), a modified Tyrode's solution with polyvinylalcohol (PVA), present to replace previously used protein supplements (e.g., bovine serum albumin [BSA]), and an atmosphere of 10% CO_2, 10% O_2, and 37.5°C supported development of in vivo-fertilized embryos to blastocysts *(55–57)*. Deletion of glucose, phosphate, and pyruvate,

reduction of lactate; and addition of glutamine *(57,58)* were important changes. Almost 30 years after the first successful IVF in golden hamsters, Barnett and Bavister *(59)* reported that IVF hamster embryos could develop in chemically defined, protein-free culture medium (HECM-3 with hypotaurine added) into morulae and blastocysts, and produce normal offspring after transfer to recipients. Inclusion of hypotaurine was essential for in vitro support of development to at least the 8-cell stage. Twenty offspring represented successful term development of 5% of the 2-cell embryos transferred into oviducts and 17% of the 8-cell embryos transferred into uteri of recipients. In contrast to chemically defined zygote culture conditions, sperm preparation and IVF were in BSA-supplemented medium *(54)*. Capacitation of cauda epididymal spermatozoa involved a 3.0–3.5-h incubation in HECM-3 with 3 mg/mL BSA, 5 m*M* glucose and phenylephrine, hypotaurine, and epinephrine (PHE) sperm-motility factors. The IVF medium also contained PHE and lowered concentrations of glucose (0.5 m*M*) and BSA (0.3 mg/mL).

Mouse

In 1968, Whittingham reported in vitro development of 65 of 159 ova (40.9%) to the 2-cell stage by 24 h after in vitro insemination with spermatozoa recovered from the uterus 1–2 h post-coitus *(60)*. Proof of fertilization included development of some in vitro-fertilized ova to 17-d-old fetuses following embryo transfer. The medium employed was a slight modification of that developed by Whitten and Biggers *(61)* to culture mouse zygotes to the blastocyst stage. Thus, progress by 1968 promised an opportunity to obtain all stages of preimplantational development in simple defined conditions.

In 1970, Cross and Brinster *(62)* reported development of 15-d-old fetuses from 3/95 mouse embryos that resulted from in vitro oocyte maturation and IVF. In their experiments, immature follicular oocytes were obtained from donors treated with pregnant mare serum gonadotropin (PMSG now referred to as equine CG) 48 h previously and inseminated with spermatozoa recovered from the uterus after mating.

A need for mouse epididymal spermatozoa to undergo capacitation was evidenced by Iwamatsu and Chang *(63)*. In Tyrode's solution supplemented with heat-treated bovine follicular fluid, the proportion of ova penetrated by fresh epididymal sperm increased from 2% at 1 h to 79% at 8 h of insemination. By contrast, epididymal sperm pretreated by incubation for 3-4 h in the same medium penetrated 17% of ova by 20 min, and 90% by 2 h after in vitro insemination *(63)*. Using a modified Krebs-Ringer bicarbonate solution containing glucose, sodium pyruvate, bovine albumin, and antibiotics Toyoda et al. *(64)* demonstrated epididymal sperm penetration to begin about 1 h (22.8% of ova penetrated) and to be completed within 2 h (96.4% penetrated) after insemination of ova. When a 2-h sperm incubation preceded insemination of ova, more than half of the ova were penetrated within 30 min, and all ova were penetrated by 1 h after insemination. Also, the mean number of penetrating sperm cells per penetrated ovum was higher (3.1) after preincubation than that (1.6) after use of fresh epididymal spermatozoa. Use of epididymal spermatozoa after increasing intervals of preincubation from 3–7 min up to 120 min resulted in increasing proportions of ova that underwent sperm penetration and early development of the fertilization process (i.e., at telophase of the second maturation division with enlarged sperm heads in the vitellus) at 1 h after insemination. Thus, capacitation of mouse epididymal spermatozoa was shown to be a progressive change that could be completed within an hour in a defined medium without factors uniquely supplied by the female reproductive tract *(64,65)*.

Miyamoto and Chang *(66)* added lactate to this medium for epididymal sperm capacitation and, following transfer to recipient female mice, demonstrated comparable fetal development of 2-cell mouse ova after IVF (13%) as that after fertilization in vivo (16%). In the same year, 1972, Mukherjee reported fertile progeny from transfer of blastocysts resulting from IVF of in vitro matured oocytes *(67)*. In these experiments epididymal spermatozoa were capacitated by in vitro incubation for 4 h at 37°C with heat-inactivated (56°C for 30 min) human follicular and tubal fluid (1:2).

A maximum rate of sperm capacitation occurred by addition of 200 m*M* NaCl to the medium used by Toyoda et al. *(64,65)* and the mechanism involved removal of a decapacitation factor *(68)*. The protein fraction eluted from previously washed epididymal sperm effected by treatment with the HIS medium decapacitated salt-capacitated sperm, and this decapacitation could be reversed by an additional incubation in the high-salt capacitating medium. Additional advances, as those reported by Hoppe and Pitts *(69)* and Fraser and Drury *(70)*, contributed to routine procedures, making mouse IVF a most useful research tool *(71)*.

Rat

Miyamoto and Chang *(72)* determined the need for rat sperm capacitation with best IVF results obtained by using spermatozoa recovered from the uterus 10–11 h after mating. Naturally ovulated ova (45%) were more readily fertilized than were superovulated (22%). In 1974, Toyoda and Chang obtained live young after transfer of 2-cell embryos that developed after direct in vitro insemination with epididymal spermatozoa *(73)*. The IVF medium consisted of modified Krebs-Ringer bicarbonate solution supplemented with glucose, sodium pyruvate, sodium lactate, BSA, and antibiotics. The time required for sperm penetration was shortened by sperm preincubation for 5–6 h and inclusion of a high potassium to sodium ratio (0.32) and 2 m*M* db-cAMP for IVF *(73)*. Under these conditions, 90% of rat ova were penetrated within 3 h after in vitro insemination.

Guinea Pig

Yanagimachi *(74)* found the medium developed for culture of early mouse embryos by Biggers, Whitten, and Whittingham (BWW medium) *(75)* to be adequate for guinea pig IVF. The time required for sperm penetration of ova was shortened from 3–4 h to 1 h after incubating fresh epididymal spermatozoa in the culture medium for 12–18 h before in vitro insemination. A close correlation between the sperm acrosome reaction and the ability to fertilize, and the necessity for calcium ions for initiation of a physiologic acrosome reaction and consequently for successful fertilization, were also reported *(74)*.

Heterologous IVF Experimentation

In 1972, Yanagimachi *(76)* showed that in vitro capacitated guinea pig epididymal spermatozoa could penetrate hamster ova freed from their zonae pellucidae, although penetration through the zona was not possible. In the same year, Hanada and Chang *(77)* reported that rat and mouse spermatozoa were also capable of penetrating zona-free hamster ova. The zona-free hamster ovum model was advocated for evaluation of capacitation, acrosome reaction, and male pronuclear development in species whose ova are difficult to directly study for any reason, e.g., human *(78,79)*, and pig *(80)*.

Slavik et al. *(81)* reported observations on developmental ability of hybrid zygotes resulting from IVF of in vitro-matured bovine ova with ram sperm. Fertilization was

successful in 83% of bovine oocytes inseminated with bull sperm (control) compared with 67% of bovine oocytes inseminated with ram sperm (hybrid embryos). In both cases, two normal pronuclei were seen and comparable development of hybrid and control embryos to the 8-cell stage resulted after transfer of 2-cell embryos to ewe oviducts. Inability of nuclei of hybrid embryos to make the maternal to embryonic genomic transition was indicated by remarkably lowered values in frequency and intensity of tritiated uridine incorporation associated with onset of RNA synthesis. The morphological observations were consistent with delay or inefficient reactivation of the embryonic genome in the hybrid embryos *(81)*.

CONTEMPORARY MOUSE IVF

The importance of laboratory mice, including transgenic and "knock-out" strains, in modern medical research has provided impetus to further development of IVF and related technologies for their efficient use. Improved culture systems for mouse embryo development *(82–85)* now provide new opportunities in murine reproduction. Much current interest surrounds cytoplasmic and nuclear transfers, new technologies that can build on advances achieved through ongoing efforts to develop IVF technology. With the advent of cloning by somatic cell nuclear transfer, (*see* Chapters 13–17) it may no longer be necessary to pursue the goal of using sperm cells as vectors for introducing foreign DNA into ova *(86)*. Incubating mouse epididymal spermatozoa with the nuclease inhibitor, aurintricarboxylic acid (ATA), enabled an increase in the yield of 2-cell IVF embryos *(87)*. Experimental results suggested the ATA acts by preserving sperm nuclei from induced or spontaneously occurring damage and/or by promoting events, apart from improved ability of sperm cells to penetrate, that trigger early embryogenesis *(87)*.

Contemporary mouse IVF with emphasis on the male gamete promises new avenues for research and for practical applications. Sasagawa et al. *(88)* demonstrated that round spermatids can fertilize following intracytoplasmic injection and artificial oocyte activation as by electrostimulation and oscillogen injection. Nuclei of primary spermatocytes have been injected into oocytes and found to undergo meiosis, form an embryo, and produce live young. It was suggested that this approach may become available as a treatment for patients with azoospermia due to maturation arrest (*88*; *see* Chapter 7). Mouse spermatozoa obtained from the epididymis from a dead animal as long as 24 h after death can be used to fertilize oocytes and the resulting zygotes can develop into live young *(89)*. The importance of more efficiently preserving valuable transgenic mouse lines has led to research emphasis on cryopreservation of sperm *(90–93)* and ova *(94)* that can later be used for embryo production via IVF. Live mice have resulted from cryopreserved embryos derived in vitro using cryopreserved ejaculated spermatozoa recovered from mated mice *(95)*. Normal offspring have resulted from mouse ova injected with spermatozoa cryopreserved with or without cryoprotection *(96)*.

Strain differences have proven to be crucial components in mouse IVF and superovulatory protocols *(97)*. Three strains commonly used in genetic engineering (ICR outbred, C57BL/6 inbred, and B6SJ1F1 hybrid) were superovulated by four different timing regimens before insemination, with spermatozoa from males of the same strain. There was a significant strain influence on IVF rate. Groups according to timing regimen, i.e., hours between PMSG (eCG) and hCG, and hours between hCG and oocyte collection, affected the proportion of fertilized ova obtained and the effect of these groups varied across mouse strain. Therefore, the treatment that produced the highest fertilization rate was

related to and contingent upon the mouse strain. This work demonstrated that responses to standardized IVF protocols vary significantly; the efficiency of IVF procedures can be optimized within specific mouse strains by the timing of superovulatory regimens; absence of cumulus cells during IVF in this work did not adversely affect fertilization rate *(97)*.

Visconti et al. *(98)* reported that it is now possible to replace BSA with synthetic cyclodextrins in media to support signal transduction leading to sperm capacitation. Data presented further support the coupling of cholesterol efflux to the activation of membrane and transmembrane signaling events leading to the activation of a unique signaling pathway involving cross-talk between cAMP and tyrosine kinase second messenger systems; this defines a new mode of cellular signal transduction initiated by cholesterol release *(98)*.

Adham et al. *(99)* reported interesting experiments with mice carrying a mutation at the acrosin locus (Acr) generated through targeted disruption in embryonic stem cells and transmitted through germ lines of chimeric male and female mice. The homozygous Acr mutant males were fertile and yielded litters comparable in number and size to Acr+/+ mice. Incubation of ova with equally mixed sperm cells of Acr+/+ and Acr–/– mice resulted in fertilization only with Acr + sperm cells. Further investigation revealed that Acr– sperm cells penetrate ova more slowly than Acr + sperm and thereby have a selective disadvantage following competitive insemination *(99)*. This work serves to emphasize the redundancy of mechanisms for achievement of discrete links comprising the chain of reproductive events necessary for procreation.

The mouse model is useful in assessing contraceptive effects as illustrated by studies with murine recombinant (r) fertilization antigen (FA)-1 used to actively immunize female mice *(100)*. The FA-1 antigen is only found in sperm/testis. A significant reduction in fertility correlating with circulating antibody titers was completely reversible with passage of time. Anti-rFA-1 antibodies from immunized mice, but not immunoglobulins from PBS-treated control mice, significantly blocked murine sperm binding to the zona pellucida and IVF of murine oocytes. These findings suggest that rFA-1 is an exciting candidate for immunocontraception *(100)*.

Mouse breeding through several generations has been useful in reproductive toxicology for many years. Burruel et al. *(101)* followed up an earlier observation linking gamma-irradiation of male mice to reduction in IVF performance 6 wk later. In this experiment, after acute 137 Cs gamma-irradiation yielding an absorbed dose of 1.0 Gy, adult cycle day (CD)1 FO males were mated weekly with CD1 females. Males from F1 litters conceived 5 and 6 wk after paternal FO irradiation were allowed to mature. Their epididymal spermatozoa were evaluated for IVF using ova from unirradiated 8–12 wk-old CD1 females. For F1 males conceived 5 wk after paternal FO irradiation, the mean fertilization rate was similar to that of the control (i.e., 80.74 ± 15.74 SD%, n = 5, and 89.40 ± 10.94 SD%, n = 8, respectively). By contrast, the fertilization rate for spermatozoa from F1 males conceived 6 wk after paternal FO irradiation (56.14 ± 21.93 SD%, n = 5) was significantly less than the fertilization rate for control spermatozoa or for that of the F1 males conceived 5 wk after paternal FO irradiation. Apparently spermatozoa obtained 6 wk after paternal FO irradiation can transmit a decrease in fertilization rate to the F1 males as well as exhibit decreased fertilization rate themselves when tested directly by IVF *(101)*.

Follicular fluid at a dilution of 10^{-4} markedly increased the proportion of mouse sperm exhibiting high velocity and stimulated chemotactic behavior as determined by tracking

on a chemotactic Zigmond chamber and recorded by videomicroscopy *(102)*. Highest sperm velocities and greater proportions of affected cells were seen with exposure to oviductal fluid. Chemotaxis was reported upon exposure of spermatozoa to oviductal fluid at dilutions of 10^{-3} and 10^{-5} and a possible sequential activity of oviductal and follicular fluids to direct spermatozoa toward ova was suggested *(102)*. Sperm-ovum chemotaxis requires further study (under rigorous conditions that can be confirmed in several appropriate laboratories) in mice and in other species. Identification of specific factors involved could prove to be very useful.

Two IVF experiments, one with zona pellucida intact and the other involving zona-free ova, were used to assess effects of a monoclonal antibody mMN9 against equatorin, a protein located at the equatorial segment of the acrosome *(103)*. The mMN9 antibody did not affect sperm motility, zona binding, or zona penetration, but it significantly inhibited fertilization. At 5 h after insemination nearly half of the unfertilized zona-intact oocytes had accumulated sperm in the perivitelline space, and by electron microscopy many unreleased cortical granules were seen beneath the oolemma indicating no sperm-oocyte fusion. Confocal laser-scanning light microscopy with indirect immunofluorescence demonstrated the presence of equatorin at the equatorial segment in capacitated and acrosome-reacted (perivitelline) sperm. These results suggest involvement of equatorin in sperm-oocyte fusion *(103)*. Addition of a synthetic peptide corresponding to a specific region of the mouse acrosomal transmembrane protein cyritestin lowered IVF rate to 30% of the normal value *(104)*. This report supports the involvement of the disintegrin domain of cyritestin in the ovum-receptor binding site of the acrosome-reacted spermatozoon. Other efforts have implicated mouse sperm fertilin alpha and beta subunits in adhesive functions relevant to sperm-ovum binding *(105,106)*.

Methods for detection of cytoskeletal and nuclear architectural structures in mouse oocytes during fertilization and methods used in exploring their regulation by intracellular calcium ion imaging have been well described by Simerly and Schatten *(107)*. These authors pointed out that methods for mammalian IVF have led to many important basic discoveries including genomic imprinting, gametic recognition involving unique receptors and galactosyl-transferases, atypical maternal inheritance patterns of the centrosome in mice, both paternal and maternal inheritance of mitochondria, and unexpected signal transduction pathways for fertilization and cell-cycle regulation. Unlike most species, the centrosome is derived from maternal sources during fertilization in the mouse (for review, *see* ref. *108*). Ultrastructural studies combined with extension of methods developed through earlier applications in the mouse model have made possible a better understanding of the fate of sperm mitochondria and sperm tail structures *(109)*, and the removal of the sperm perinuclear theca and its association with the oocyte surface during bovine fertilization *(110)*.

Initial success in growing preantral mouse oocytes from secondary follicles coupled with in vitro oocyte maturation (IVM) and IVF to obtain offspring was reported by Eppig and Schroeder in 1989 *(111)*. Subsequently, complete oocyte development was achieved by Eppig and O'Brien *(112)*. Primordial follicles were first allowed to develop in newborn mouse ovarian culture for 8 d to become secondary follicles. Then methods developed earlier were applied, i.e., culture of isolated oocyte-granulosa cell complexes for 14 d followed by IVM and IVF. Development from 2-cell to blastocyst stages was around 2%. Transfer of 198 2-cell stage embryos resulted in 2 offspring. One died shortly after birth and the survivor had impaired health that led to early death (*112*; for review, *see* ref. *113*).

DEVELOPMENT OF IVF IN DOMESTIC ANIMALS

Bovine

Among domestic species, greatest progress in developing IVF and complementing technology has resulted from emphasis on bovine reproduction (for reviews, *see* refs. *114,115*). As with development of IVF procedures in laboratory animals a major challenge involved development of sperm treatments for ovum penetration. Initial success was facilitated by models made available by rabbit in vitro capacitation and through studies of bull sperm interaction with zona-free hamster ova (for review, *see* ref. *116*). In 1977, initial success with IVF and comparison with in vivo fertilization and early cleavage development was documented by ultrastructural observations *(117)*. This involved insemination of in vivo matured ova with high ionic strength-treated ejaculated bull spermatozoa. In the same year, Iritani and Niwa *(118)* reported penetration and activation of around 20% of follicular oocytes following maturation of the oocytes for 20–24 h at 37°C in modified KRB. This oocyte treatment had been found earlier to result in approx 60% development to metaphase II *(119)*.

Sperm penetration, as observed 19–24 h after in vitro insemination, was favored by ova recovered from follicles and/or oviducts near the time of induced ovulation when compared with those recovered from unstimulated 2–5 mm follicles and cultured for 18–25 h before insemination *(120)*. Such observations point to sperm-penetration influencing factors associated with female gametes. Ultrastructural studies revealed loss of cortical granules and presence of sperm remnants in ova and embryos resulting from in vivo and in vitro fertilization *(121)*. Extension of these efforts led to publication in 1982 of a repeatable procedure for bovine IVF, with documentation including the first IVF calf born in June, 1981 *(122)*. In vivo matured ova were retrieved after treatment of donors with prostaglandin $F_2\alpha$ followed by PMSG (eCG). In one experiment, frozen-thawed semen was successfully employed, but usually fresh semen was incubated, high ionic strength-treated, and subsequently incubated in DM prior to insemination of ova. A range of fertilization results was observed according to different bulls that provided semen; spermatozoa from one bull fertilized 22 (62.9%) of 35 tubal ova. In vitro development proceeded to the 8-cell stage. Vigorous progressive sperm motility and acrosome integrity were important features of good sperm samples capable of penetrating zona-free hamster ova and cow oocytes *(122)*.

Initial success evidenced by live calves after transfer of IVF embryos into recipients involved surgical procedures for ovum recovery and transfer *(122,123)*. Improvement in the procedure followed laparoscopic recovery of follicular oocytes from hormonally treated cows just before ovulation without impairment of the donor's fertility *(124–127)*. Sirard and Lambert *(128)* found 68% of the recovered oocytes to be mature and approx one-half of them could be fertilized upon incubation with capacitated spermatozoa from selected bulls.

Variability in obtaining suitable ova following hormonal treatments, difficulties inherent in directly working with the animals, and high costs led scientists to devote serious attention to maturing oocytes easily obtained from small ovarian follicles at slaughter. In vitro matured slaughterhouse oocytes represent valuable experimental material for developing improved treatments for capacitation and the acrosome reaction. Proteoglycans and glycosaminoglycans (GAGs) are among the capacitating agents present at the nor-

mal site of fertilization in vivo (for reviews, *see* refs. *129,130*). By 1986, Parrish et al. had described an effective heparin treatment to enable cryopreserved bull spermatozoa to fertilize in vitro *(131)*. The glycosaminoglycan, heparin, has been extensively studied *(132,133)* and is most widely used in bull-sperm treatments to effect in vitro capacitation. Interestingly, relationships are now recognized between the degree of affinity for heparin by heparin-binding proteins (HBPs) in spermatozoa *(134)* and in patterns of binding of seminal vesicle-derived HBPs on the sperm surface *(135)*, with bull fertility in artificial insemination (AI) use. Although heparin/heparan sulfate is the group of GAGs implicated as most likely to be responsible for physiologically effecting capacitation in estrual cows *(136)*, available evidence also implicates other classes of GAGs, bicarbonate, calcium, and complementary roles for many additional factors in the physiological preparation of spermatozoa for fertilization.

Hyaluronic acid (HA) has been suggested as a capacitating agent for bull sperm *(137)*. Swim-up procedures through columns of HA-supplemented media have enabled selection of viable, fertile bull spermatozoa for use in IVF *(138,139)*. By traversing the HA-containing medium, the sperm surface is subjected to high shearing forces that might remove decapacitation factors thereby contributing to the capacitation process. An unrelated positive influence involving supplementation of chemically defined culture media with HA (1 mg/mL) was recently found to significantly enhance bovine embryo development to the blastocyst stage after IVM and IVF *(140)*.

In addition to those mentioned previously, successful approaches employed in preparing bull sperm for IVF include a synergistic combination of heparin and caffeine *(141)*, brief treatment with the calcium ionophore A23187 with or without caffeine in DM *(26)*, Percoll separation followed by incubation with hypotaurine or caffeine *(142,143)*, and calcium-free Tyrodes at pH 7.6 *(144)*.

For frozen-thawed spermatozoa of (at least) some bulls, it is possible to greatly simplify conditions with defined media for in vitro capacitation *(145,146)*. Thus, with completely defined media (i.e., protein-free, with PVA replacing BSA in modified DM) for in vitro capacitation, IVF and embryo culture, caffeine (1.0 mg/mL), and penicillamine (0.5 mg/mL) were adequate to replace the usual addition of heparin in the capacitation treatment for optimal results. Thus, of immature oocytes selected for IVM 76, 69, and 63% reached morula, blastocyst and expanded blastocyst stages, respectively, under these conditions. Resulting embryos appeared morphologically normal with a mean of 91 cells at 216 h post insemination. Further evidence of viability followed challenge of 96 late morulae and early blastocysts (144 h post-insemination) by vitrification. After thawing, 67% had survived as determined at 72 h and 82% of these reached the expanded blastocyst stage by 96 h of culture *(146)*.

In similar chemically defined IVF medium but with elevated bicarbonate, Tajik and Niwa *(147)* subjected cumulus-free ova to spermatozoa of 5 different bulls to study effects of caffeine (5 m*M*) with and without heparin (10 μg/mL) and interactions with glucose (13.9 m*M*). Sperm penetration took place only in the presence of caffeine and/or heparin. Regardless of the presence of glucose, similar proportions of ova were penetrated in the heparin-containing medium with (73 and 83%) and without (36 and 41%) caffeine, but with caffeine alone a higher penetration rate was observed in the presence (41%) than in the absence (27%) of glucose. When ova inseminated in medium containing caffeine and heparin with or without glucose were cultured in a chemically defined, protein-free

medium, 90 and 72% reached at least the 2-cell stage (by 48 h) and 21 and 9% reached blastocyst stages (by 192 h post-insemination), respectively. In these conditions, glucose was beneficial and caffeine and heparin synergism was not explained by caffeine's reversal of glucose inhibition of heparin-induced capacitation *(147)*.

A part of bull-sperm treatments for IVF involves selection of the most progressively motile sperm cell populations for insemination. This is commonly done by allowing the cells to swim up into media over an interval of 30–60 min after washing the sperm cells by centrifugation. Another useful means for accomplishing this has involved centrifugation of spermatozoa through a gradient of 45 and 90% Percoll. Parrish et al. *(148)* found cleavage rates after IVF to be significantly higher with swim-up selected spermatozoa, than after Percoll separation. Comparable cleavage and blastocyst rates were also reported after IVF with frozen-thawed bull spermatozoa separated with glass wool and by swim-up techniques *(149)*. Another interesting approach, transmigration (TM) advocated by Rosenkranz and Holzmann *(150)*, selects motile spermatozoa in a target chamber after swimming from a test chamber through a unipore membrane (with 8 μm diameter pores) against a stream of capacitating medium (5 mL h^{-1}). Using this system, bull-ejaculated spermatozoa did not need any additives, as heparin, hypotaurine, and epinephrine, to promote capacitation and to increase motility *(151)*. Using a TALP medium *(152)* supplemented with pyruvate, BSA, heparin, hypotaurine, and epinephrine, spermatozoa from TM samples penetrated 39.3% of ova by 5 h post-insemination, whereas with swim-up samples first instances of penetration were observed only 2 h later (4.2%). By this time a normal-looking pronucleus was observed in 16.4% of ova in the TM group. Polyspermy was seen only after 7 h of incubation. This nonstatic method may provide a better means for avoiding sperm penetration of ova that become aged along with accompanying abnormal fertilization that may be more common when swim-up separated spermatozoa are used *(150)*.

Complete functional oocyte maturation—including nuclear, cytoplasmic, and membrane components—is necessary for the ooplasm to promote male pronuclear development and to launch ongoing mitotic development of the new individual after fertilization (*see* Chapter 3). Many experiments during the past two decades have revealed positive influences on ovum quality as judged by improved embryonic development following in vitro insemination. Addition of luteinizing hormone (LH) (100 μg/mL) to serum-containing medium for IVM enabled development in culture to 8- to 16-cell stages without extraneous cells for co-culture, and pregnancies could be initiated with the resulting IVF embryos following transfer *(153)*. Earlier, to avoid subjecting recently cleaved IVF embryos to inadequate culture conditions, laparoscopic oviductal transfer was shown to facilitate term development after IVM and IVF *(154)*. By 1993, undefined conditions incorporating selected serum, hormones, and other active agents, and cellular constituents or their contributions to support in vitro production of bovine embryos enabled achievement of IVF (cleavage) of 85% and blastocyst development of 40% of oocytes selected for IVM (for review, *see* ref. *155*). These efforts along with realization that IVM was best supported by certain cow sera, proestrous *(156)* or estrous *(157,158)*, that contained high LH concentrations led to replacement of serum with purified bovine LH (USDA-bLH-B-5) for supplementation of modified tissue culture medium (TCM)-199 to provide defined conditions for IVM *(159,160)*. Defined conditions for IVM coupled with semidefined conditions (i.e., with purified BSA present) for IVF and cell-free embryo culture enabled IVF of 74% and blastocyst development of 28% of selected immature oocytes *(155)*.

It seems likely that part of the beneficial effect of high concentrations of biological preparations of gonadotropins may reflect presence of biologically active contaminants, e.g., activin A *(161–163)*, and/or growth hormone *(164)*. Epidermal growth factor (EGF) *(165)* and insulin-like growth factor-1 (IGF-1), *(166–168*), act synergistically with gonadotropins to improve oocyte, and resulting embryo quality. Combination of follicle stimulating hormone (FSH) and platelet-derived growth factor (PDGF) or EGF ± PDGF during IVM also enhanced blastocyst development *(169)*. Transforming growth factor-α (TGF-α), binds to the EGF receptor and similarly enhances bovine IVM *(170)*. Further refinement of conditions for IVM include replacement of biological gonadotropin preparations by human recombinant gonadotropins *(171,172)*. Optimal blastocyst production followed inclusion of 10 ng/mL r-hFSH in the Medium 199-based defined milieu for IVM *(172)*. This enabled 73.9, 41.3, 33.7, and 25.0% development of ova to cleavage, morula, blastocyst, and expanded blastocyst stages, respectively, in completely chemically defined conditions.

Much contemporary research involves improvement of in vitro culture media to support development to blastocyst stages (for reviews, *see* refs. *173,174* and Chapter 8). Interleukin-1 (IL-1) added at 8–10 h after insemination (at 0.1–1 ng/mL) increased development to the blastocyst stage when embryos were cultured at high density (~25–30/drop) but had no effect on development when cultured at low density (~10/drop) suggesting involvement of some other embryo-derived product *(175)*. The effect of IL-1 on embryonic development was maintained in completely denuded embryos, indicating that cumulus cells do not mediate the actions of IL-1. The effective treatment also increased embryo cell number at d 5 post-insemination by increasing the proportions of embryos that reached the 9- to 16-cell stage, but IL-1 had no effect on the proportion of blastocysts when added at d 5 post-insemination *(175)*. Several growth factors, e.g., PDGF, EGF, IGF-1, have been shown to be efficacious in promoting morula to blastocyst stages (for review, *see* ref. *176*). Protection from oxidative damage by maintaining a physiological O_2 tension (i.e., 5–8%) and lowered initial glucose concentration *(177)* and by bolstering intracellular glutathione by inclusion of mercaptoethanol or cysteamine *(178)* have had positive influences on blastocyst development after IVF.

Proportions of blastocysts resulting from bovine IVF procedures reflect not only the composition of media for support of IVM, sperm preparation, IVF (i.e., in vitro insemination), and in vitro culture (IVC) but interactions between treatments for each biological step constituting the entire IVMFC system. Also, embryos develop better in less dilute conditions due to beneficial influences of autocrine/paracrine factors. The optimal volume in culture drops is related to changes in medium *(179)* which, in turn, must be balanced against build-up of ammonium ions or other toxic by-products of embryo metabolism (for review, *see* ref. *173*). Modifications of synthetic oviduct fluid, SOF *(180)* with glutamine or citrate and nonessential amino acids (g-SOF + NEA or c-SOF + NEA) *(181)* and with BSA replaced by PVA have proven effective for support of zygote to blastocyst stages after IVF, with IVMFC in completely defined media *(146,172)*. Culture conditions were improved by use of a more complex TCM-199-based medium with recombinant growth factors for support of morula to blastocyst stages *(168)*. The ability to produce bovine embryos in completely defined conditions should facilitate advances in optimization of proportions of immature oocytes that can become viable, transferrable embryos. Such defined conditions will also be of great importance in control of disease.

Porcine

Cheng et al. *(182)* reported the first successful IVF piglets. Mattioli et al. *(183)* developed an oocyte maturation procedure incorporating the whole wall of everted follicles. Earlier difficulties in obtaining normal male pronuclear development were overcome after insemination with Percoll-separated spermatozoa, and blastocyst formation, pregnancies and birth of live piglets after IVF were reported *(184)*. The presence of cumulus cells is important in stabilizing the distribution of cortical granules to maintain the ability of the oocyte to undergo sperm penetration *(185)*. Mattioli et al. *(186)* found choline uptake, unlike uridine uptake, by LH-treated cumulus-intact oocytes was significantly higher than in FSH-treated or control oocytes. After 44 h of maturation culture, the percentage of oocyte reaching metaphase II was significantly higher in the presence of LH (76%) and FSH (86%) than in the controls (35%). The percentage of oocyte supporting male pronuclear formation was 48.4% in the control and 44.3% in FSH-treated oocyte, whereas 72.7% of LH-treated oocyte supported male pronuclear development. The observation that LH selectively increases the uptake of choline suggested that changes in metabolic coupling during maturation are not only quantitative but also qualitative, probably accounting for the regulatory effect of the cumulus cells. Mattioli et al. *(187)* demonstrated influences of LH on cyclic AMP (cAMP) and the ability of cAMP to maintain meiotic arrest, or for a transient increase in cAMP to facilitate meiosis (*see* Chapter 3). Funahashi et al. *(188)* applied these concepts to increase homogeneity of oocyte nuclear maturation by exposing cumulus oocyte complexes to dibutyryl cAMP for the first 20 h of culture. The incidence of embryos that developed to the blastocyst stage after IVF was higher following exposure to dbcAMP (21.5 vs 9.2% for controls). After transfer of experimental embryos to four recipient gilts, three delivered 19 live piglets *(188)*.

Harrison *(189)* has emphasized the importance of bicarbonate in affecting the architecture and functioning of boar spermatozoa. Recent work on membrane changes provoked by cooling has indicated similarities with capacitational changes and therefore cooling may induce premature capacitation (and destabilization). Initiation of premature capacitation by cryopreservation has been demonstrated for bull spermatozoa *(190)*. Studies of sperm-zona interaction using cryopreserved cumulus-free immature oocytes led to conclusion that the strength of attachment reflected the area of sperm head in contact with the zona rather than any physiologically specific binding, and zona attachment was not a functional or temporal indicator of zona penetration *(191)*.

Wang et al. *(192)* investigated combination of 10% pig follicular fluid and cysteine (0.57 m*M*, 1:1) with NCSU 23 medium, TCM-199, or modified Whitten's medium for IVM and found no differences in nuclear maturation, cortical granule distribution, sperm penetration, male pronuclear formation, polyspermy, and cleavage in oocytes matured in the three media. However, the NCSU 23-based medium gave significantly better results in glutathione content, cortical granule exocytosis, blastocyst development (30%), and number of cells in blastocysts (36.8 ± 17.0), indicating improvement in cytoplasmic maturation of porcine oocytes *(192)*. When follicular shell pieces were included for IVM, significantly higher proportions of blastocysts were obtained than following IVF of ova matured in absence of follicular shell pieces *(193)*. Ova matured with pieces of follicular shells also had higher glutathione concentrations. Resulting embryos were viable as demonstrated by term development *(193)*. In recent work, porcine ova were successfully matured in a protein-free medium with subsequent development to the blastocyst stage

(194). In other efforts glucocorticoids were shown to directly inhibit the meiotic, but not cytoplasmic, maturation of porcine ova in vitro *(195)*.

Investigation of morphological characteristics of in vitro matured vs in vivo matured ova revealed differences with in vitro ova exhibiting greater cytoplasmic density in the cortex, thinner zonae that were less resistant to pronase digestion, and lacking affinity for a lectin specific for beta-D-Gal (1-3)-D-GalNAc *(196)*. Although comparable in ability to release cortical granules on sperm penetration, the polyspermy rate was significantly higher for in vitro matured oocytes (65%) than for ovulated oocytes (28%). The results suggested oviductal alterations in functional blocking of polyspermy in porcine ova *(196)*. Morphological evaluation of embryos produced in vitro vs in vivo revealed abnormalities in 27% of 2-cell, 74% of 3-cell, 51% of 4-cell, and 74% of 5- to 8-cell IVF embryos *(197)*. Abnormalities (not seen in in vivo embryos) included fragmentation and/or binucleation. Staining by rhodamine-phalloidin with confocal microscopic examination revealed fewer or no perinuclear actin filaments in blastomeres of IVF embryos. There were significantly more cells in d 5 (136.5 ± 60.4 nuclei per blastocyst) and d 6 (164.5 ± 51.9 nuclei per blastocyst) in vivo blastocysts than were present in d 6 in vitro-produced blastocysts (37.3 ± 11.7 nuclei per blastocyst). These results implicate abnormal actin filament distribution in abnormal cleavage and resultant small numbers of cells in porcine embryos produced in vitro *(197)*.

Ovine

The first lambs resulting from ovine IVF were reported in 1986 by Cheng et al. *(182)*. Ten offspring were delivered by 7 of 16 recipients after transfers 16 h after in vitro insemination. Crozet et al. *(198)* achieved IVF with ovulated and in vitro matured ova and a lamb was born from one of the ovulated ova. Ultrastructural study of oocytes 20–24 h after insemination indicated that IVF approximated in vivo events. Temporal development of fertilization was identical to in vivo development reported earlier. Sperm incorporation into ooplasm was seen as early as 2 h post-insemination; abstriction of the second polar body, by 3–4 h post-insemination; male and female pronuclear formation, by 5 h post-insemination; and development of the first mitotic spindle was seen at 21 h post-insemination *(199)*. Impairment of cytoskeletal function was likely caused by a high incidence of polyspermy (27% of penetrated ova) as reflected in retarded or absent pronuclear migration *(199)*.

Additional efforts were reported by Fukui et al. *(200,201)*. Marked differences in IVF were recognized according to individual rams providing spermatozoa. Elevation of the calcium concentration in the modified DM fertilization medium was found to stabilize the fertilization rate afforded by various rams *(202)*. These conditions led to fertilization of 82.6% of ovulated ova and 61.7% were monospermic *(203)*. Transfer of oocytes taken 17 h after insemination resulted in pregnancies and offspring.

Recent progress by Wang et al. *(204)* demonstrated significant enhancement of oocyte maturation by FSH and LH as compared to hCG alone. Ova fertilized in vitro with spermatozoa treated with calcium ionophore A23187 and caffeine in DM provided significantly greater embryonic development in vitro than when swim-up separated spermatozoa in synthetic oviduct fluid supplemented with estrus ewe serum was used *(205)*. Their IVF procedure was also documented by birth of lambs. Ovine IVF has also been used recently to examine differences between sheep with and without the Booroola gene *(205)*. Other

experiments have shown that induction of the acrosome reaction in spermatozoa before intracytoplasmic sperm injection is unnecessary, whereas a capacitating treatment of spermatozoa is required before IVF *(206)*.

Caprine

In 1985, Hanada *(207)* reported the birth of a kid resulting from IVF of in vivo matured oocytes with A23187-treated spermatozoa. In vitro-capacitated epididymal spermatozoa fertilized goat follicular oocytes in early work of Song and Iritani *(208)*. Modified DM was found to be superior to TALP or modified H-M199 for caprine-sperm capacitation and better results were obtained with IVM, than with oocytes harvested after hormonal treatments of does *(209)*. After IVM with a high concentration of LH, 39.5% of the oocytes were fertilized and three pregnancies were initiated after oviductal transfer of 2- and 4-cell IVF embryos *(209)*. DeSmedt et al. *(210)* obtained IVM to metaphase II of 86% of oocytes from 2–6-mm diameter follicles but only 24% for oocytes from 1–2 mm follicles. Using their capacitation treatment for ram spermatozoa, this group obtained a high fertilization rate but also found polyspermy in almost 20% of inseminated ova. By 41 h after insemination, 58% of the IVM-IVF ova reached 2- and 4-cell stages. Comparable results were also reported by Chauhan and Anand *(211)*.

By 1994, conditions were described for IVM, IVF, and IVC (IVMFC) enabling immature oocyte to develop into morulae *(212)* and to kids after embryo transfer *(213)*. Procedural improvements allowed consistent in vitro development to the blastocyst stage, and successful uterine transfers of morulae after IVF with birth of normal offspring *(214)*. Oocyte cumulus complexes were matured during 27 h in TCM-199 supplemented with 20% fetal bovine serum (FBS), 100 μg LH/mL, 0.5 μg FSH/mL, and 1 μg estradiol 17-β/mL at 38.5ºC in a humidified 5% CO_2, 5% O_2, and 90% N_2 atmosphere. Freshly collected spermatozoa were washed and incubated for 5 h in mDM-containing 20% FBS, then treated with 7.35 m*M* calcium lactate in the presence of ova for 14 h, followed by IVC on a cumulus-cell monolayer in HEPES buffered TCM-199 with 10% FBS under paraffin oil. Thirty microliters of spent medium was replaced by an equal volume of fresh HM-199 every 24 h. These conditions supported development of 31.8% inseminated ova to the blastocyst stage *(214)*.

In further work, the feasibility of using frozen-thawed semen in caprine IVF outside the breeding season was demonstrated *(215)*. The highest proportion of blastocysts (35.7% of oocytes inseminated) resulted from use of spermatozoa diluted in a skim milk extender, heparin capacitation, and insemination in mDM supplemented with lamb serum *(215)*. In this work the defined IVC medium used for bovine zygotes (i.e., c-SOF + NEA) *(181)* proved effective for caprine IVC.

Poulin et al. *(216)* reported that out of the breeding season a higher polyspermic fertilization rate (41% vs 13–15%) occurred when 5 μg heparin/mL vs 0.2 and 1 μg/mL, respectively, was included in the fertilization medium; the latter consisted of modified DM with 20% heat-inactivated estrous sheep serum. Only 18% of cleaved ova developed to the blastocyst stage in the 5 μg/mL heparin group compared to 40% in the other groups. In the breeding season the blastocyst yield from cleaved ova was similar with or without heparin in the IVF medium, but after transfer of 2 blastocysts per recipient 8/9 recipients gave birth to 11 kids (developmental rate of 61%) in the control group and only 5/10 gave birth to 5 kids (developmental rate of 25%) in the heparin group. Poulin et al. *(216)* concluded that limited success in producing live offspring in the goat with IVM-IVF ova could be due to use of heparin as a capacitating agent for buck spermatozoa.

An extension of efforts to produce caprine embryos out of the normal breeding season involved development of a procedure for intracytoplasmic sperm injection (ICSI). A significant improvement in fertilization and blastocyst development over the IVF control was achieved by this approach. Thus, oocytes can be obtained from does out of the normal breeding season, matured in vitro, fertilized by injection of frozen-thawed spermatozoa (after manipulation to break their tails), and cultured to blastocysts (25% of oocytes) in defined medium *(217)*.

Equine

In 1991, Palmer et al. *(218)* reported the first foal derived by IVF, and Bezard et al. *(219)* reported the second the following year. In this work preovulatory oocytes were fertilized by calcium ionophore-A23187-treated stallion spermatozoa. Most IVF efforts in the literature with in vivo or in vitro matured oocytes have resulted in development of pronuclear or early cleavage stages, but conditions for oocyte maturation, sperm capacitation, and embryo culture are not well developed to accommodate in vitro embryo production in this species. Greatest progress has followed implementation of procedures for ICSI. In 1996, Squires et al. *(220)* reported the first pregnancy after equine ICSI. By ICSI, Dell'Aquila et al. *(221)* obtained 2 pronuclei or cleavage of 29.8% (17/57) vs 8.7% (9/103) of slaughterhouse oocytes inseminated in vitro. Similarly, fertilization was reported following ICSI in additional efforts *(222,223)*. Recently pregnancies have also followed injection of frozen-thawed sperm cells into in vivo matured oocytes *(224)* and ICSI involving oocytes obtained from pregnant mares *(225)*. Squires et al. *(226)* pointed out the potential utility of ICSI for assessing the viability of variously treated oocytes and the need for improved culture systems. Only 7% of 204 sperm-injected oocytes developed beyond the 8-cell stage although around 40% cleaved at least once *(227)*.

Canine

Efforts to achieve IVM of canine oocytes collected at random stages of the estrous cycle led to germinal vesicle breakdown of only 24.5% of the oocytes within 72 h *(228)*. Following insemination with dog sperm incubated for 7 h to effect capacitation, oocytes at any stage could be penetrated *(228,229)*. Improved results followed use of preovulatory oocytes recovered from ovaries of beagle bitches after hormonal treatment *(230)*. After culture for 72 h in modified Krebs-Ringer bicarbonate supplemented with 10% FCS approx 32% of oocytes reached metaphase II, and by 8 h following insemination with 4-h preincubated dog spermatozoa male and female pronuclei were seen. Sperm penetration of the zona pellucida began around 1 h post-insemination. Transfer of oocytes at 18–20 h after insemination into Whitten's medium enabled cleavage by 48 h post-insemination. Development of 15 of 45 inseminated oocytes to 2- to 8-cell stages was reported *(230)*. Successful term development following canine IVF has not been reported. Interest in canine IVF technology extends beyond applications for pets to include several endangered species *(231)* including the gray wolf (Canis lupis), the red wolf (Canis rufus), the Simien fox (Canis simensis), the San Joquin kit (Vulpes macrotis mutica), and the northern swift fox (Vulpes velox hebes).

Feline

Initial successful IVF in cats resulted from insemination of oviductal ova with uterine spermatozoa recovered after mating to assure capacitation *(25)*. Additional evidence for IVF followed use of spermatozoa obtained from the epididymis and ductus deferens

(232,233). Goodrowe et al. *(234)* reported the birth of kittens following oviductal transfer of 2- to 4-cell stage IVF embryos. Oocytes were recovered by laparoscopy after PMSG (eCG) and hCG treatments and spermatozoa were collected by electroejaculation. Sperm capacitation included a swim-up procedure. The IVF success rate was highly dependent on the time interval between PMSG and hCG treatments as well as the hCG dose. Overall fertilization (48.1%) and cleavage (45.2%, at 30 h post-insemination) rates were highest following an 80 h PMSG to hCG interval combined with a 100 IU hCG dose. Five of 6 cats receiving 6–18 embryos became pregnant and produced 1–4 kittens/litter. The gonadotropin-treated queens subjected to follicular aspiration produced normal corpora lutea as judged visually at embryo transfer and by circulating progesterone values similar to control cats *(234)*. This work has provided a useful basis for extension to non-domestic endangered Felidae.

Pope et al. *(235)* classified cumulus oocyte complexes according to their cytoplasmic morphology as type A = good, type B = fair, and type C = poor, and matured them for 24 h in TCM-199 with gonadotropins (eCG, FSH, hCG ,or FSH/hCG). Frequency of cleavage after IVF for type A (54%), B (41%), and C (26%) oocytes was similar to the IVM frequency of the equivalent type. Development of morulae was similar among types (47–58%), but higher percentages of types A and B reached blastocysts, 31 and 29%, respectively, than of type C (15%). Transfer of d 6 (n = 32) morulae and blastocysts to three d 5 recipients resulted in pregnancies and birth of 4 live kittens *(235)*.

Kanda et al. *(236)* compared several media and culture types and achieved their best results when follicular ova were fertilized and cultured in modified Earle's balanced salt solution (MK-1) supplemented with 10% human serum (HS); the fertilization rate was 94.7% and 50% reached the blastocyst stage. When the same medium was used in a suspension culture dish (in contrast to a tissue culture dish), 47.2 and 71.7% of IVF-derived embryos developed to blastocysts at 120 and 144 h post-insemination, respectively. When 6 embryos per cat were transferred to the uterine horns of recipients 8 of 10 recipients that received early blastocysts (120 h) became pregnant *(236)*.

APPLICATIONS OF IVF

Research

Recently developed IVF technology offers unprecedented opportunities for research to improve mammalian reproductive efficiency and to understand basic mechanisms involved in early development. An important avenue involves comparative studies of gene expression for assessment of normal development. The effects of a semi-defined culture system on temporal mRNA expression patterns of 10 developmentally important genes analyzed in bovine oocytes and embryos produced in vitro were recently reported *(237)*. The transcriptional pattern assessed by reverse transcription-polymerase chain reaction (RT-PCR) of IVF morulae and blastocysts was compared with that of their in vivo counterparts. Compared with in vivo-derived embryos, bovine embryos derived from IVF, in general, appear different by light and electron microscopy, differ in number of cells, size, developmental rate, temperature sensitivity, freezability, viability, and pregnancy rates after transfer *(238)*. Earlier, Wrenzycki et al. *(239)* discovered a qualitative difference at transcription. Post-implantation events influenced by culture conditions are thought to be involved in the delivery of abnormally large fetuses or offspring after transfer of bovine embryos grown in vitro *(240)*. The most prominent correlation

is that the high serum content of the media is a major relevant factor *(241)*. Genes analyzed by Wrenzycki et al. *(237)* were chosen to characterize effects of a widely used bovine IVF system on physiological processes involved in compaction and cavitation, glucose metabolism, RNA processing, stress, and early differentiation. Evidence for expression of gap junction protein connexin 43 (Cx43) was detected in blastocysts derived in vivo but not in those derived in vitro. This was not due to a difference between serum and BSA supplementation of media *(237,238)*. Characterization of influences of improved chemically defined conditions for in vitro embryo production in this way should be valuable in efforts to produce physiologically normal embryos (*see* Chapter 8).

Additional avenues of research are opening with further development of microinjection approaches building on current IVF technology, e.g.. bovine ICSI *(242–245)*. Much can be learned of fertilization, appropriateness of gamete preparation, and of cytological events through further development of these potentially useful research tools.

Animal Breeding

Bovine embryos resulting from IVF have already received much commercial interest *(246–248)*. A major boon to practical applications followed development of transvaginal oocyte retrieval capabilities introduced by Pieterse et al. in 1988 (*249*; for review, *see* ref. *250*). In vitro production (IVP, also used as abbreviation for in vitro produced) of bovine embryos may enable replacement of currently practiced artificial insemination since direct embryo transfer after cryopreservation should prove to be a more efficient means for pregnancy initiation. The latest statistics reported by the International Embryo Transfer Society noted over 30,000 IVP embryos were transferred into recipient cows in 1997 *(251)*. Bousquet et al. *(252)* combined IVP with embryo sexing *(253)* and embryo transfer in a commercial setting. This group demonstrated that IVF procedures can effectively replace conventional in vivo embryo production methods when a predetermined number of pregnancies of known sex are needed within a short interval of time *(252)*. Also, bovine IVF provides a means to decrease the generation interval *(254–256)*; to produce calves via sexed semen *(257)*; to overcome infertility *(252)*; to expand reproductive potential of pregnant animals *(258)*; to extend reproductive life; to assist in propagation of endangered cattle breeds *(259)*; to produce large numbers of half-siblings simultaneously; to extend valuable semen via sperm injection *(242,260)*; to assess gamete performance; to provide pronuclear ova for DNA microinjection *(261)*; and to provide the framework for a variety of gamete manipulations including cytoplasmic transfer, nuclear transfer *(262)*, and cloning by blastomeric recycling *(263)*.

Opportunities for utility of IVF are promised by recent advances in preservation of functionality after cryopreservation of embryos (for review, *see* refs. *264,265*), and of bovine oocytes (*266,267*; *see* Chapter 10*)*. All of the aforementioned should gain impetus from further optimization of chemical and physical conditions for IVP of embryos. This can be facilitated through use of currently available chemically defined conditions for bovine IVM, IVF, and IVC *(146,171,172,176)*.

Prediction of porcine fertility by homologous in vitro penetration of immature oocytes enabled discrimination between boars used for AI characterized as low (<20%), intermediate (40–60%) and high (>80%) fertility groups *(268)*. Parameters of motility, normal morphology, normal apical ridge, viability with eosin-nigrosin stain, hypo-osmotic swelling test, osmotic resistance test, and functional membrane integrity with carboxyfluorescein diacetate were useful in detecting sperm with poor fertility but not precise enough

to discriminate between an ejaculate with higher fertility than the herd median. Only the penetration percentage (10.24 ± 1.45 vs 55.13 ± 3.35 vs 84.72 ± 1.73) and sperm number per oocyte (1.29 ± 0.07 vs 11.29 ± 1.79 vs 25.86 ± 1.43) were parameters with a predictive capacity to discriminate between the three fertility groups *(268)*. Another approach to assessing semen quality involved IVF of in vitro matured oocytes *(269)*. In this test, all measures of sperm fertilizing ability were different among boars (all $p < 0.05$) and use of different semen dilutions for IVF allowed further discrimination of apparent sperm quality among boars. For all semen dilutions, estimated potential embryo production rate accounted for up to 70% of the variation in litter size obtained with $3 \leftrightarrow 10^9$ sperm per AI dose, and the number of sperm attached per oocyte was a major factor accounting for variation in litter size obtained with $2 \leftrightarrow 10^9$ sperm per AI dose *(269)*. These IVF variables hold promise as indicators of boar sperm quality for use in AI. Successful implementation of IVM-IVF with X- and Y-bearing spermatozoa using USDA sperm-sexing technology included surgical embryo transfers and birth of piglets of predetermined sex *(270)*. Recent development of a nonsurgical approach for embryo transfer in pigs *(271)* can be anticipated to further enhance practical uses of porcine IVF technology.

Many of the practical applications in development for farm animals are already feasible for mice. Thus, IVF offers much to efforts to make procreation possible or more efficient in a variety of research circumstances.

Preservation of Endangered Species

In the past 200 years more than 50 mammalian species have vanished and over 200 species are currently being threatened by extinction. The need for extending IVF to scarce gametes of endangered species is obvious but species differences have imposed major technological barriers (for review, *see* ref. *272*). The potential of IVF for conservation of endangered mammalian species is nonetheless tremendous (for review, *see* ref. *273*). Success has been attained using IVF to produce offspring in the Indian desert cat *(274)* and in the Siberian tiger *(275)* as well as several species of non-human primates. Experimentation with zebra led to IVF of 38% of oocytes and 16% development to morula or blastocyst stages *(276)*. Progress in understanding fertilization mechanisms can be obtained through heterologous IVF, as was recently demonstrated with spermatozoa from endangered African antelope, the scimitar-horned oryx, and domestic cow oocytes *(277)*. Cow oocytes were fertilized by spermatozoa from all oryx males tested, and 34 (61.8%) of 55 2-cell embryos produced developed to at least the 8-cell stage; polyspermy was detected in 7 (29.2%) of 24 uncleaved oocytes *(277)*.

CONCLUSIONS AND FUTURE PROSPECTS

Progress in IVF is evidenced by the ability to achieve gamete union and pregnancies following embryo transfer in common laboratory and domestic animals. Additionally, defined conditions are available for support of oocyte maturation, sperm capacitation, IVF, and embryo culture for several species. These and other advances in complementing technologies provide great impetus for acceleration of additional refinements in IVF to afford better ways to enhance reproductive efficiency and to understand physiological events at the molecular level.

Greater prominence can be anticipated for IVF in research and practical applications. The facility to produce embryos by combining previously cryopreserved gametes (*see* Chapter 10) will be useful in breeding of laboratory, domestic, and zoo animals. Technol-

ogy developed in the course of animal IVF experimentation should provide a good basis for further advances, especially ICSI (*see* Chapter 7), cloning (*see* Chapters 13–17), and improved means for genetic engineering in animals. Refinements in animal IVF systems promise better ways to test for contraceptive development and models for improving assisted human reproduction.

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